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Interrelationships between Colonies, Biofilms, and Planktonic Cells of *Pseudomonas aeruginosa*▽†

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Pseudomonas aeruginosa **is a gram-negative bacterium and an opportunistic human pathogen that causes chronic infections in immunocompromised individuals. These infections are hard to treat, partly due to the high intrinsic resistance of the bacterium to clinically used antibiotics and partly due to the formation of antibiotic-tolerant biofilms. The three most common ways of growing bacteria in vitro are as planktonic cultures, colonies on agar plates, and biofilms in continuous-flow systems. Biofilms are known to express genes different from those of planktonic cells, and biofilm cells are generally believed to closely resemble planktonic cells in stationary phase. However, few, if any, studies have examined global gene expression in colonies. We used a proteomic approach to investigate the interrelationships between planktonic cells, colonies, and biofilms under comparable conditions. Our results show that protein profiles in colonies resemble those of planktonic cells. Furthermore, contrary to what has been reported previously, the protein profiles of biofilms were found to more closely resemble those of exponentially growing planktonic cells than those of planktonic cells in the stationary phase. These findings raise some intriguing questions about the true nature of biofilms.**

Pseudomonas aeruginosa is a gram-negative, aerobic bacterium that is ubiquitous in the environment and causes chronic biofilm infections in immunocompromised individuals (5, 10). These infections are notoriously difficult to treat due to the high resistance of biofilms to antibiotic intervention (19, 23). A commonly used definition of a biofilm is a "microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription" (11). Intuitively, these criteria should encompass most cell aggregates, including bacterial colonies on agar plates; however, very few studies have mentioned bacterial colonies in a biofilm context. A large number of experimental setups have been employed to study biofilms in vitro (7, 8, 34–37), and these experiments have provided a wealth of information on biofilm formation and life cycle. However, extrapolations from these experiments to clinically relevant biofilms in vivo have proven difficult.

In a biofilm, cell densities are substantially higher than in planktonic culture (24). As a consequence, most biofilm cells are likely to encounter nutrient and oxygen limitation as well as higher levels of waste products, secondary metabolites, and secreted factors (22, 37). Because of this, it is not surprising that gene expression in sessile cells is very different from that in their free-floating counterparts. *N*-Acyl homoserine lactone-mediated cell-cell signaling termed quorum sensing, which in planktonic cultures coordinates bacterial behavior in a cell density-dependent manner, has also been shown to be important in biofilm formation (22).

Several studies have highlighted the differences in gene expression levels between biofilms and planktonic cells (12, 25, 30, 34–36). However, there are large discrepancies between the studies, and the small number of differentially expressed genes that all (or most) of them have in common has caused several workers to question the existence of a "universal biofilm phenotype" (3, 18, 34). Interestingly, the quest for a universal phenotype has so far been restricted to cells in biofilms, whereas no universal planktonic cell phenotype has been defined to date. Some of the reasons for discrepancies between the studies appear to be related to the method of analysis (transcriptomic [12, 35, 36] versus proteomic [25, 34] studies). The lack of "experimental gold standards" (3), the lack of a clearly defined "endpoint" in biofilm experiments (18), differences in thresholds and coverage, and the intrinsic heterogeneity of biofilms may also influence the results. Such heterogeneity is by no means restricted to biofilms. Colonies on agar plates can display a high level of differentiation, and stationary phase in a planktonic culture is merely a descriptive term which is likely to cover a heterogeneous population of cells in different metabolic states (17). Even exponentially growing cells of both *Escherichia coli* and *Bacillus subtilis* have been shown to be heterogeneous (2, 15). The results of most global studies therefore represent an average of results for populations with greater or lesser degrees of heterogeneity.

The majority of in vitro experiments are performed on either planktonic cells, colonies on agar plates, or biofilms in continuous-flow systems. However, the following questions arise: what is the relationship between these three modes of growth, and is it at all possible to extrapolate between them? It has been suggested that biofilm cells resemble planktonic cells in stationary phase (32), and several studies have presented data to support this view (12, 31, 35). However, none of these studies mention colonies on agar plates. These colonies could

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be predicted to behave like biofilms, since they are organized communities encased in a polysaccharide matrix with high cell densities and coordinated cellular behavioral patterns (28). Indeed, complex colony architecture has been used as an analogy for biofilm formation in other species, such as *B. subtilis* (6, 9, 20).

Here we ask the question, what is the interrelationship between planktonic cells, biofilms, and colonies on agar plates? Because proteins are the dominant functional entities in the cell, we chose to investigate this on the protein level. We employed two-dimensional fluorescence difference gel electrophoresis (2D-DiGE), coupled with biological variance analysis (BVA), which allows direct quantitative comparison of proteins across multiple gels. Our results show that, under our growth conditions, the protein profiles of colonies are similar to those of planktonic cells. Unlike in previous studies, we also find that biofilms in a constant-flow system are more similar to exponentially growing cells than to stationary-phase cells.

MATERIALS AND METHODS

Strain and growth conditions. *Pseudomonas aeruginosa* PAO1 was obtained from Barbara Iglewski, University of Rochester (Rochester, NY). All cells were grown at 37°C in AGSY medium (56 mM alanine, 17 mM K_2HPO_4, 86 mM NaCl, 100 μM $CaCl_2$, 10 mM $MgSO_4$, 5 μM $FeCl_2$, 7.5 μM $ZnCl_2$, 0.5% [vol/vol] glycerol, 3 g/liter yeast extract, pH 7). Planktonic cells were grown in baffled flasks with vigorous shaking and harvested after 3 h (late exponential phase; optical density at 600 nm [OD_{600}], ~1) or 9 h (stationary phase; OD_{600}, ~9). Colonies were grown on 1.5% AGSY agar. After 15 or 40 h, cells were harvested by flooding the plates with ice-cold TE lysis buffer (50 mM Tris-HCl, 4 mM EDTA, pH 8.3) and scraping off the colonies with a glass spreader. After 15 h, the colonies were white and flat (0.5- to 1-mm diameter), whereas after 40 h they were pigmented and differentiated (5- to 7-mm diameter). Biofilms were inoculated with 1 ml of an overnight culture (OD_{600} diluted to 1) and grown in silicone tubes (15-cm length, 6-mm internal diameter) with a continuous flow of 50 ml per hour (variation, ≤5%). This corresponds to laminar flow (Reynolds number 3.3). The tubes were placed vertically in order to avoid sedimentation of planktonic cells. Biofilms were grown for 3 or 5 days and harvested by squeezing out the accumulated material. In this growth medium, the 3-day-old biofilms represented the earliest point at which we could reliably obtain sufficient material for proteomic analysis. After this, the biofilms grew rapidly and were expected to have reached maturity by day 5. By day 6, the biofilms often began to disperse and peel off from the inside of the tube. All cells were sedimented by centrifugation (10 min, 3,000 × *g*, 4°C) and stored at −80°C. The biofilm pellets were larger after 5 days than after 3 days, but there was no significant difference in the numbers of CFU.

Protein extraction. Pellets were resuspended in TE lysis buffer supplemented with 0.1 mM phenylmethylsulfonyl fluoride and Complete Mini-Protease inhibitor cocktail (Roche). Cells were disrupted by sonication on ice, and debris was removed by centrifugation (30 min, 13,000 × *g*, 4°C). Proteins were extracted with phenol using an adaptation of the method described by Hurkman and Tanaka (13). TE-saturated phenol (1 volume) was added to the cleared cell lysates; samples were vortexed five times for 1 min each time and kept on ice for 1 min between the rounds. The layers were separated by centrifugation (10 min, 1,600 × *g*), and the top aqueous layer was discarded. Phenol-saturated TE (2 volumes) was added, samples were vortexed and centrifuged as before, and the aqueous layer was discarded. We found that this phenol extraction step was crucial in order to achieve consistent reproducible separation of *P. aeruginosa* proteins on the 2D gels. Proteins were precipitated by adding 5 volumes of 100 mM ammonium acetate in 100% methanol and leaving the samples at −20°C overnight. Pellets were washed, first with 80 mM ammonium acetate in 80% methanol and second with 80% acetone, and then air dried. Proteins were redissolved in ASB14 buffer (8 M urea, 2% [wt/vol] amido sulfobetaine 14, 5 mM magnesium acetate, 20 mM Tris-HCl, pH 8.5), quantified using the Bio-Rad DC protein assay kit by following the supplier's protocol, and adjusted to a total protein concentration of 5 mg/ml with ASB14 buffer.

Proteomics and data analysis. We analyzed four biological replicates for each of the six conditions examined, i.e., 24 samples in all (14). Proteins were minimally labeled using charge neutral CyDye DIGE Fluor minimal dyes (GE Healthcare). Two samples from each of the growth conditions were labeled with Cy3 and two with Cy5 in order to avoid potential dye-related artifacts. A pooled internal standard consisting of equal amounts of all 24 protein samples was generated and adjusted to a final protein concentration of 5 mg/ml with ASB14 buffer. The standard was labeled with Cy2 and included in all gels. This ensured that all proteins were represented on every gel, allowing direct inter- and intragel comparison of protein spots. Furthermore, this procedure also made it possible to distinguish between spots that were absent because of differences in the biological samples or because of technical differences between the gels. Fifty micrograms of each sample or pooled internal standard was labeled with Cy3, Cy5, or Cy2 (200 pmol) for 30 min, and the labeling reaction was quenched by adding lysine (50-fold excess). Following this, samples were combined (details available upon request) so that each gel contained two samples (one labeled with Cy3 and one with Cy5) and a Cy2-labeled standard. The first dimension was run on 24-cm isoelectric focusing DryStrips (pH 4 to 7; GE Healthcare), and the second dimension was run on Ettan DALT 12% sodium dodecyl sulfate-polyacrylamide gels. Gels were scanned at the appropriate wavelengths for excitation of Cy3, Cy5, and Cy2 using a Typhoon 9400 scanner, and the resulting 36 images (3 for each of the 12 gels) were analyzed using the DeCyder software package (GE Healthcare). A typical image of a Cy2-labeled internal standard was chosen as a master gel to which spots from the remaining 11 Cy2-labeled images were matched using DeCyder BVA 5.02. Following this, a univariate BVA was performed as previously described (1, 33) using Student's *t* test ($P \leq 0.01$). Based on data generated by DeCyder BVA, a principal-component analysis (PCA) was performed using SimcaP 10.0 (Umetrics). PCA utilizes the BVA output data. However, unfiltered BVA data may contain noise that can easily be excluded by visual inspection in the univariate analysis but which, if not removed, can interfere with multivariate analysis. Therefore, the data were filtered prior to PCA such that only those spots which were resolved in at least 75% of all gels and had a pixel intensity of $\geq 1 \times 10^4$ were included. The filtered data set was also subjected to a hierarchical cluster analysis (HCA) using Pearson's correlation in Java TreeView 1.0.12 (jtreeview.sourceforge.net). Preparative gels were loaded with 200 μg of unlabeled pooled internal standard and then fixed (45% methanol, 1% acetic acid) and stained with Coomassie blue. Proteins of interest were excised and subjected to tryptic digestion and analyzed by liquid chromatography (LC)-tandem mass spectrometry (MS-MS). Peptides were separated by reverse-phase nano-high-performance LC (CapLC pump from Waters; 15-cm PepMap C_{18} column with a 75-μm inside diameter from LC Packings) on a capillary LC system attached to a model QTof2 mass spectrometer (Waters). Peptides were eluted with a flow rate of 2.95 μl/min in a gradient of 5 to 55% acetonitrile over 33 min and 55 to 95% over 5 min. The MS-MS fragmentation data were used to search the NCBI database (www.ncbi.nlm.nih.gov) using the MASCOT search engine (www.matrixscience.com).

RESULTS

In the current study, an analysis was performed on protein extracts from three different growth modes harvested at two different time points. Hereinafter, these protein extracts will be referred to using the following abbreviations: PE, PS, C15h, C40h, B3d, and B5d. P, C, and B refer to planktonic cells, colonies, and biofilms, respectively. Planktonic cells were harvested in exponential phase (E) or stationary phase (S); colonies were harvested after 15 or 40 hours of incubation, and biofilms were harvested after 3 or 5 days (3d or 5d) of growth.

Biological variance analysis and Student's *t* test. Between 1,279 and 1,606 protein spots in the Cy2-labeled standards were matched to the master gel, an image of which is shown in Fig. S1 in the supplemental material. Following gel-to-gel matching, each protein spot (with a minimum of three data points for each of the relevant conditions) was analyzed for significant differences between the six groups of samples using Student's *t* test with a threshold set at a *P* of ≤0.01 (Table 1). The highest number of significant changes (358 spots) was found in a comparison of PE and PS. The smallest number of differences was found between B3d and B5d (46 spots), followed by PE and C15h (106 spots). Furthermore, PS samples were more similar to C40h than to any other group of samples.

TABLE 1. Numbers of spots that were significantly modulated between the six conditions tested

Condition 1 sample	No. of spots significantly ($P \leq 0.01$) modulated (no. of up-regulated proteins, no. of down-regulated proteins) compared with those in condition 2 sample[a]:				
	B5d	B3d	C40h	C15h	PS
PE	175 (101, 74)	148 (87, 61)	314 (191, 123)	106 (69, 37)	358 (205, 153)
PS	322 (138, 184)	323 (134, 189)	159 (72, 87)	235 (94, 141)	
C15h	143 (69, 74)	137 (62, 75)	209 (115, 94)		
C40h	260 (99, 161)	251 (107, 144)			
B3d	47 (16, 31)				

[a] For example, 175 spots were modulated between PE and B5d samples, of which 101 were more abundant in PE and 74 were less abundant in PE.

Biofilms, irrespective of age, were more similar to PE and C15h than to PS and C40h. This is in contrast to previous studies that have suggested a high degree of similarity between biofilms and stationary-phase planktonic cells (12, 35).

PCA and HCA. In order to validate the results from the univariate analysis, we employed two multivariate analytical techniques. PCA is frequently used to identify patterns in data of high dimension in situations where graphical representation is not readily available, whereas HCA sorts samples into groups based on degree of similarity. The analyses were unsupervised, meaning that the calculations were performed without any information on the nature of the samples. The filtered data set used for multivariate analyses (see Materials and Methods) contained 1,072 variables, with 24 observations for each.

First, PCA was used to identify the largest variations in the data set. This analysis is based upon the generation of a covariance matrix describing how each spot varies with respect to every other spot in the data set. Eigenvectors, along with their corresponding eigenvalues, can be calculated for the covariance matrix, describing correlated variation within the data set. The eigenvector with the highest eigenvalue represents the largest variation in the data set and is the first principal component (PC1). Similarly, the eigenvector with the second-highest eigenvalue is PC2 and so on. In this way, the complexity inherent in this high-dimensional data set can be reduced to a relatively small number of ranked orthogonal PCs (29). A score plot of PC1 versus PC2, which accounted for 48% of the total variation in the data set (29% for PC1 and 19% for PC2), clustered samples from the six different groups (Fig. 1). PC1 separated PS and C40h from the other samples. The effect of the incubation time in planktonic cells and colonies therefore appeared to account for the largest variation in the conditions studied. PC2 separated biofilms from both planktonic cells and colonies, indicating a different protein profile in these samples. Interestingly, the biofilm samples were closer to the actively growing PE and C15h than to PS and C40h samples.

In order to confirm the clustering of samples, especially regarding those that appeared to be closely related, the same data set was subjected to an HCA (Fig. 2A). This analysis confirmed that the major split in the dendrogram was between biofilms, PE and C15h on one hand and PS and C40h on the other hand. The analysis also showed that the biofilms had expression profiles that were different from those of both colonies and planktonic cells and that these were more closely related to PE and C15h than to PS and C40h.

Protein identification. Between PE and PS, approximately one-third of the reliably detected proteins were found to be modulated (358 spots out of 1,072 detected in ≥75% of the samples [Table 1]). Furthermore, the PCA plot (Fig. 1) indicated that the effects of the incubation time were similar in planktonic cells and in colonies. Indeed, of the 209 proteins that were modulated between C15h and C40h, 122 were also found to be modulated between PE and PS, and 118 of these changed in the same direction. However, PC2 indicated that there were differences between B3d, PE, and C15h other than those related to incubation time. Between these three groups, as well as between PE and PS, we attempted to identify those proteins which exhibited the highest change (*n*-fold). Some protein spots yielded a list of peptides that matched more than one protein in the *P. aeruginosa* database. This could be due to spot comigration or incomplete separation of proteins on the gels. In the case of such mixed hits, we were unable to account for which of the two or more proteins was responsible for the observed modulation, so these have been excluded from further discussion. Single hits, along with their level of change (*n*-fold) between selected conditions are presented in Fig. 2B. Furthermore, a selection of modulated proteins had been identified in a previous study under the same experimental conditions, and these are also shown in Fig. 2B. All single hits are presented in Fig. 2B, and a list of these proteins with the corresponding MS-MASCOT data are shown in Table S2 in the supplemental material.

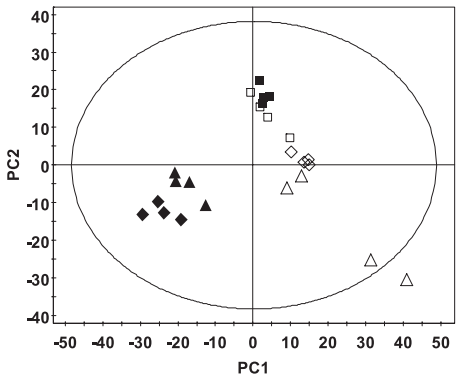
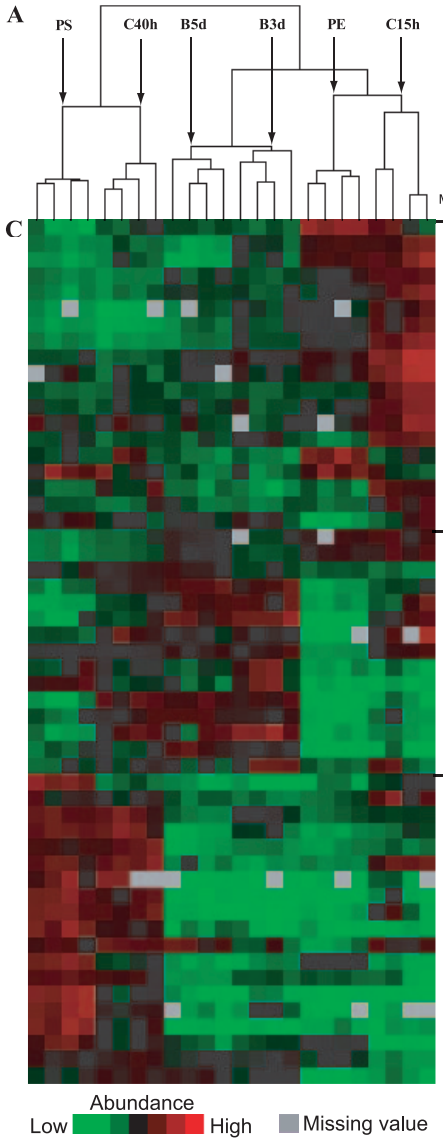


FIG. 1. PCA score plot of PC1 versus PC2. PC1 accounted for 29% of the variation in the data set, whereas PC2 accounted for another 19%. Shown are results for planktonic cells in exponential phase (◇) or in stationary phase (◆), colonies incubated for 15 h (△) or 40 h (▲), and biofilms incubated for 3 days (□) or 5 days (■).



† Iron-regulated (21)
‡ Biofilm-specific and quorum-sensing regulated (12)
× Quorum-sensing and RpoS-regulated (27)
¤ Modulated in biofilms *vs*. planktonic cells (12)

B

Master No.	PA number	Gene name	Gene product	Proteins significantly modulated in BVA (p≤0.01) B3d(C15h)	B3d(PE)	PE(C15h)	C40h(C15h)	B5d(B3d)	PS(PE)	Pearson's Correlation Coefficient	Category
1394	0865	*hpd*	4-hydroxyphenylpyruvate dioxygenase	-	-6.30*	-	-	-	-11.98*		1
376	2624	*idh*	isocitrate dehydrogenase	-2.64	-1.96	-	-3.81	-2.62*	-3.89	0.96	
397	2624	*idh*	isocitrate dehydrogenase	-3.82	-2.19	-	-4.51	-3.94*	-5.19		
502	5016	*aceF*	dihydrolipoamide acetyltransferase	-2.98	-1.64	-1.81	-4.75	-	-2.42	0.91	
730	4385	*groEL*	GroEL protein	-3.02	-	-	-4.85	-	-2.4		
556	3162	*rpsA*	30S ribosomal protein S1	-	-	-	-8.76*	-	-2.87		
567	3162	*rpsA*	30S ribosomal protein S1	-2.44	-	-1.95	-8.25	-	-3.00	0.93	
564	3162	*rpsA*	30S ribosomal protein S1	-3.73	-	-2.74	-9.00*	-1.59	-2.47		
1245	4238	*rpoA*	DNA-directed RNA polymerase alpha chain	-3.86	-	-3.34	-8.29*	-	-1.87		
938	3134	*gltX*	glutamyl-tRNA synthetase	-4.46	-	-3.34	-6.20	-	-		
871	3770	*guaB*	inosine-5'-monophosphate dehydrogenase	-4.02	-	-3.68	-3.35	-	-	0.84	
1034	5554	*atpD*	ATP synthase beta chain	-3.14	-	-2.59	-3.10	-	-		
1026	5554	*atpD*	ATP synthase beta chain	-3.61	-	-	-	-	-		
233	0572		hypothetical protein PA0572	-2.16	2.19	-4.72*	-3.55	-	-		
409	0459		probable ClpA/B protease ATP binding subunit	-2.33	-6.39*	2.74	-	-	-3.10		
1391	4277	*tufB*	elongation factor Tu	-	-3.97	-	-	-	-		
1458	1777	*oprF*	major porin and structural outer membrane porin OprF precursor	-5.30*	-	-3.65	-	-	-		
1467	1777	*oprF*	major porin and structural outer membrane porin OprF precursor	-2.96	-	-2.75	-	-	-		
475	3790	*oprC*	Putative copper transport outer membrane porin OprC precursor	-	4.65	-4.84*	-	-	5.89		
445	4761	*dnaK*	DnaK protein	-1.98	-1.76	-	-2.46	1.50	-3.38	0.98	2
451	4761	*dnaK*	DnaK protein	-2.32	-1.88	-	-2.77	1.62	-3.29		
372	0482	*glcB*	malate synthase G	1.71	-	-	2.76	1.63	1.79		
1478	5427	*adhA* ¤	alcohol dehydrogenase	2.30	6.23*	2.71	1.69	-	1.99		
740	4236	*katA*	catalase	-	5.79	-	-	-	-	0.86	
2119	4067	*oprG* ¤	outer membrane protein OprG precursor	-	22.05*	-11.24*	-	-	-		
1307	5172	*arcB*	ornithine carbamoyltransferase, catabolic	-	9.01*	-20.24*	-	-	5.07	0.85	
2136	3326		probable Clp-family ATP dependent protease	-	4.06	-4.04	-	-	2.93		
1571	0852	*cbpD*	chitin-binding protein CbpD precursor	5.41*	6.04	-	2.71	-	3.82	0.86	
1634	0852	*cbpD*	chitin-binding protein CbpD precursor	9.34*	11.42*	-	3.99	-	9.68		
1543	4352	¤	conserved hypothetical protein PA4352	4.57	12.51*	-	5.48	-	-		
2280	3309		conserved hypothetical protein PA3309	-	8.17*	-	-	-	-	0.81	
2277	5267	*hcpB*	secreted protein Hcp	8.22*	16.56*	-	3.69	-	-		
2320	1900	*phzB2*	probable phenazine biosynthesis protein	4.40	7.58*	-	-	-	4.19		
2077	5139		hypothetical protein PA5139	-	3.53	-	-	-	-		
346	4710	*phuR* †	Haem/Haemoglobin uptake outer membrane receptor PhuR precursor	-4.80*	-	-2.52	-	-	8.24		3
923	2413	*pvdH* †	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase, PvdH	-4.33	-	-3.45	-	-	2.27		
517	0588	×	conserved hypothetical protein PA0588	-	1.79	-	2.72	-2.10*	3.78		
529	0588	×	conserved hypothetical protein PA0588	-	2.68	-2.14	3.88	-3.82*	7.56	0.91	
1624	1135		conserved hypothetical protein PA1135	-	-	-	4.19	-1.76	4.37		
1084	4470	*fumC1* †‡	fumarate hydratase	-6.55*	-	-5.09*	-	-	4.93		
2033	1013	*purC*	phosphoribosylaminoimidazole-succinocarboxamide synthase	-	-	-	-	-	15.79*		
1840	2395	*pvdO* †‡	PvdO	-	-	-	-	-	22.36*	0.93	
1780	2396	*pvdF* †‡	pyoverdine synthetase F	-	-	-	-	-	11.13*		
1116	1092	*fliC*	flagellin type B	-	-	-	10.31*	-	14.28*		
1901	1821		probable enoyl-CoA hydratase/isomerase	-	-	-4.16	-	-	4.34		
2019	5153		probable periplasmic binding protein	-	-2.64	3.62	3.01	-	2.83		
1921	3397	*fpr* †‡	ferredoxin–NADP+ reductase	-	-	-	3.77	-1.99*	3.10		
2330	3785		conserved hypothetical protein PA3785	-	-	-	8.72*	-	40.94*	0.95	
2491	4922	*azu*	azurin precursor	-	-	-	-	-	20.73*		
1994	3931	‡	conserved hypothetical protein PA3931	-	-	-	3.95	-	10.53*		
1343	3190		probable binding protein component of ABC sugar transporter	6.75*	-	8.76*	9.58*	-	2.18		
1516	3195	*gapA*	glyceraldehyde 3-phosphate dehydrogenase	2.83	-	2.55	3.98	-	-		
1598	1789		hypothetical protein PA1789	2.91	5.27	-	4.13	-	5.09		

FIG. 2. (A) HCA of data from DeCyder BVA based on the complete filtered data set of 1,072 discrete variables. (B) Proteins identified as modulated between selected conditions. All proteins identified as "single hits" are displayed. The master number is assigned by the DeCyder software and refers to the position of the protein spot on the master gel (Fig. S1 in the supplemental material). An overview of the identified proteins and the corresponding MS-MASCOT data are shown in Table S2 in the supplemental material. The *n*-fold change refers to the first condition compared with the one in brackets; e.g., negative values in the column headed by "PS(PE)" refer to a down-regulation in PS compared with expression in PE. Proteins that are among the 15 spots with the highest change (*n*-fold) in the conditions compared are marked with an asterisk. In all cases, the changes are significant, with a P of ≤0.01. Pearson's correlation coefficient indicates the correlation between variables or groups of variables and is shown for the most similar expression profiles. A hierarchical cluster analysis of protein spots in the full data set divided them into three categories, which are separated by bold lines. (C) Heat plot of the identified proteins showing the variation in expression profiles across all 24 samples.

In order to group the identified proteins into categories, an HCA was performed on the filtered data set of 1,072 protein spots. This analysis segregated the proteins into three main categories (Fig. 2B). Pearson's correlation coefficient is given for the most similar expression profiles, and expression patterns of the identified proteins across all 24 samples are shown in a heat plot (Fig. 2C). The three main categories were characterized by (i) proteins that were highly expressed in C15h and PE, (ii) proteins that were highly expressed in biofilms, or (iii) proteins that were highly expressed in PS and C40h. In category 1, the majority of the proteins were related to metabolism, transcription, and translation. Category 3 consisted mainly of proteins previously identified to be regulated by quorum sensing, RpoS, or both. Category 2 comprised proteins involved in diverse physiological processes, ranging from metabolism, adaptation/protection, and attachment through to secreted factors. Furthermore, two Usp-type stress proteins, PA4352 and PA3309, which have been previously shown to be induced under anaerobic conditions (4, 26), were highly expressed in biofilms and C40h. This could indicate an anaerobic environment in these growth modes.

DISCUSSION

Numerous studies have investigated the relationship between planktonic cells and biofilms, and large differences have been reported between their two modes of growth. However, most of the previous studies have excluded an analysis of colonies on agar plates, and many of them have included only one growth phase of the planktonic cells. In this study, planktonic cells in exponential phase, as well as in the stationary phase of growth, were used as a reference point to get an overview of the global relationships between planktonic cells, colonies, and biofilms.

Using the proteomic technique 2D-DiGE coupled with BVA, we show that the largest variation in the conditions studied corresponds to the transition between planktonic cells in exponential and in stationary phase. Colonies appear to undergo a similar transition between 15 and 40 h of incubation. Biofilms, on the other hand, do not appear to express a "stationary-phase" protein profile, even after 5 days of growth. The results from the proteomic analysis and BVA were validated with two independent multivariate approaches, PCA and HCA. A score plot of PC1 versus PC2 (Fig. 1) placed PE close to C15h and PS close to C40h. This plot also confirmed that biofilms are more closely related to PE than to PS. Using HCA, a dendrogram that further supports the results from BVA and PCA was generated (Fig. 2A).

A hierarchical clustering of the protein spots divided them into three major categories (Fig. 2B). With consideration of the effect of incubation time (C40h versus C15h and PS versus PE), planktonic cells and colonies have many proteins in common, and these are in all cases modulated in the same direction. The majority of proteins expressed in exponential phase are clustered at the top of category 1. Similarly, category 3 is dominated by proteins expressed in the stationary phase, and several of these have previously been shown to be regulated by quorum sensing, RpoS, or iron levels (12, 21, 27). Biofilms exhibit few significant changes between 3 and 5 days of growth. This is probably because the two incubation times represent two stages of maturity rather than a developing and a mature biofilm. Interestingly, the majority of proteins in category 3, which in this system characterizes the stationary phase, have very low expression levels in biofilms. This questions the common belief that there is a connection between the biofilm lifestyle and life in the stationary phase.

Looking at the differences between PE, C15h, and B3d, category 1 shows higher levels of growth-related proteins in C15h than in B3d. This is consistent with the results in Fig. 1 showing that C15h/PE and C40h/PS occupy opposite ends of the score range, whereas biofilms are more centrally located. On the other hand, in category 3, there is no overall trend between PE, C15h, and B3d, indicating that these proteins are involved mainly in the stationary-phase lifestyle. In category 2, most proteins are highly expressed in biofilms, and apart from DnaK and GlcB, all proteins in this category have significantly higher expression levels in B3d than in PE. Some of these proteins are also induced in PS compared with in PE, but their expression is higher in biofilms than in stationary-phase planktonic cells. Furthermore, the modulated proteins in category 2 appear much more diverse than the other categories and can be divided into several subgroups (Fig. 2B). For example, the expression profiles of AdhA, KatA, and OprG are closely related, judging by Pearson's correlation coefficient. These proteins are highly expressed in biofilms, moderately expressed in colonies, and expressed only at a very low level in planktonic cells. This suggests that these proteins might be involved in processes associated with high cell densities, such as detoxification. Similarly, ArcB and PA3326 are moderately expressed in biofilms, colonies, and PS but much less so in PE. A third subgroup consists of the secreted protein HcpB and the conserved hypothetical proteins PA4352 and PA3309, which have previously been shown to be induced under anaerobic conditions (26). These proteins are expressed in biofilms and C40h, indicating an anaerobic environment.

In conclusion, to our knowledge, this study is the first to investigate the interrelationship between planktonic cells, colonies, and biofilms using a global approach under comparable conditions. The results raise some intriguing questions about the true nature of biofilms, as well as our definitions of them. Are they really phenotypically distinct entities, or are both biofilms and colonies simply variants in a continuum of growth modes? Our data show, as has been suggested by Donlan and Costerton, that bacterial colonies behave like planktonic cells "stranded" on a surface (11). However, in a recent review, Kolter and Greenberg describe a *P. aeruginosa* colony as an air-exposed biofilm (16). Our results also indicate that continuously fed biofilms—at least under our growth conditions—are much more metabolically active than planktonic cells in the stationary phase. In this context, it would be interesting to see whether static biofilms on a liquid-air interface undergo a transition into stationary phase similar to the transition of planktonic cells and colonies when nutrients are limited. Also, is antibiotic resistance in biofilms the result of a "true" biofilm phenotype, or is it a product of multiple environmental factors? Current work in our laboratories is aimed at investigating these questions.

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